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A c-Jun Dominant Negative Mutant Protects Sympathetic Neurons against Programmed Cell Death

Jonathan Ham,* Carol Babij,* Jonathan Whitfield,* Curt M. Pfarr,† Dominique Lallemand,† Moshe Yaniv,† and Lee L. Rubin*
*Eisai London Research Laboratories
University College London
London WC1E 6BT
United Kingdom
†Unité des Virus Oncogènes
Centre National de Recherche Scientifique
Département des Biotechnologies
Institut Pasteur
75724 Paris Cedex 15
France

Summary

Sympathetic neurons depend on nerve growth factor (NGF) for survival and die by apoptosis in its absence. We have investigated the pattern of expression of the Jun and Fos family of transcription factors in dying sympathetic neurons using antibodies specific for each family member. When sympathetic neurons are deprived of NGF, the level of c-Jun protein significantly increases, whereas the levels of the other members of the Jun and Fos family remain relatively constant. c-Jun also becomes more phosphorylated, probably on its amino terminal transactivation domain. When microinjected into sympathetiic neurons, an expression vector for a c-Jun dominant negative mutant protects them against NGF withdrawal-induced death, indicating that AP-1 activity is essential for neuronal cell death. Furthermore, overexpression of the full-length c-Jun protein is, in itself, sufficient to induce apoptosis in sympathetic neurons.

Introduction

In multicellular organisms, cell death by apoptosis occurs during normal development, tissue homeostasis, and the response to viral infection and plays an important role in all of these situations (Ellis et al., 1991; Raff, 1992; Raff et al., 1993). In the case of the developing mammalian nervous system, cell death is widespread, with approximately 50% of the neurons produced by neurogenesis dying by apoptosis (Oppenheim, 1991). These programmed cell deaths (PCD) are regulated by factors, such as the neurotrophins, that promote the survival of specific populations of neurons (Levi-Montalcini, 1987; Barde, 1989; Snider, 1994). According to the neurotrophin hypothesis (for a recent discussion, see Korsching, 1993), the neurons that innervate a particular target are produced in excess and compete for limiting quantities of survival factor, which is produced in minute amounts by their target. Those neurons that do not obtain an adequate supply of survival factor die by apoptosis. This is thought to be a mechanism for correctly matching the number of innervating neurons with the size of their target.

Sympathetic neurons isolated from the superior cervical ganglion (SCG) of the rat have proved to be a particularly useful model system for studying PCD in the nervous system (Martin et al., 1988). During the development of the rat, all SCG neurons are postmitotic at birth, and 35% of these die between days 3 and 7 (Wright et al., 1983). SCG neurons can be isolated from P1 rats and will survive for extended periods in culture in the presence of nerve growth factor (NGF). When deprived of NGF, they die by apoptosis, and the majority of the cells are dead after 3 days (Martin et al., 1988; Edwards et al., 1991; Deckwerth and Johnson, 1993; Edwards and Tolkovsky, 1994). An important observation was made by Martin et al. (1988), who discovered that inhibitors of transcription or protein synthesis greatly reduced the rate at which sympathetic neurons die in the absence of NGF. This result led to the hypothesis that the removal of survival factor induces a gene or set of genes encoding proteins that trigger or cause cell death (for a review, see Johnson and Deckwerth, 1993). Furthermore, a requirement for transcription and protein synthesis has proved to be a general feature of neuronal cell death induced by the withdrawal of survival signals and has been observed with several different types of neuron isolated from both the central and peripheral nervous systems (Oppenheim et al., 1990; Scott and Davies, 1990; D' Mello et al., 1993; Milligan et al., 1994). The observation that neuronal cell death and certain other types of PCD, such as the activation-induced death of T cells (for references, see Liu et al., 1994 and Woronicz et al., 1994), require transcription and protein synthesis led to the notion that apoptosis is an active, gene-directed process, and that apoptotic cells actively participate in their own demise. However, it should be noted that transcription and protein synthesis are often not required for cells to undergo apoptosis (Martin, 1993; Jacobson et al., 1994), suggesting that cell death effector proteins must preexist in many types of cells.

We are investigating the molecular mechanisms underlying mammalian neuronal cell death. In particular, we would like to understand the basis for the transcriptional requirement. For example, what genes are activated when sympathetic neurons are deprived of NGF? How are these genes regulated, and how do their products lead to cell death? As an initial step, we have chosen to investigate the pattern of expression of the Jun and Fos family of transcription factors. Since members of the Jun and Fos family have already been demonstrated to play important roles in cell proliferation and differentiation (Angel and Karin, 1991; Curran and Vogt, 1992), it seems likely that in some cell types they might also regulate the expression of genes involved in cell death. Indeed, there is already evidence that certain transcription factors, such as c-Myc and E2F, which were previously thought to be primarily involved in promoting cell proliferation, can also cause cell

death when overexpressed in serum-starved, quiescent fibroblasts (Evan et al., 1992; Wu and Levine, 1994; Qin et al., 1994). Furthermore, an association between the expression of a c-Fos–β-galactosidase fusion protein and certain forms of neuronal cell death has already been noted in c-*fos*–*lacZ* transgenic mice, but whether this was a cause or consequence of death was not determined (Smeyne et al., 1993).

There are four members of the Fos family, c-Fos, Fos B, Fra 1, and Fra 2, and three members of the Jun family, c-Jun, Jun B, and Jun D, which bind as Jun/Jun or Jun/Fos dimers to the AP-1 binding site, or TPA response element (TRE; consensus sequence TGA C/G TCA) and which collectively constitute the activity known as AP-1 (Angel and Karin, 1991; Curran and Vogt, 1992). The transcription of the c-*jun* and c-*fos* genes is activated by a variety of signal transduction pathways, usually as an immediate early response. In addition, the activity of the Jun and Fos proteins may be directly regulated by phosphorylation, proteolysis, or oxidation/reduction (Abate et al., 1990; Hirai et al., 1991; Karin, 1994; Treier et al., 1994). c-Jun and c-Fos activate transcription efficiently and can transform cells in culture. In contrast, the other members of the Jun family, Jun B and Jun D, are weaker transactivators and can antagonize c-Jun (Chiu et al., 1989; Hirai et al., 1989; Schütte et al., 1989; Deng and Karin, 1993; Schlingensiepen et al., 1993; Pfarr et al., 1994). In the case of the Fos family, it has been suggested that Fra 1 and Fra 2 might function as negative regulators, since they are weak activators of transcription and cannot transform rodent fibroblasts (Wisdom and Verma, 1993). Changes in the relative levels of the different members of the AP-1 family can have important biological consequences (see, for example, Schlingensiepen et al., 1993 and Pfarr et al., 1994), which emphasizes the importance of considering all of the family members when studying the role of AP-1 in a particular biological context.

Here, we have used antibodies specific for each member of the Jun and Fos family to investigate their pattern of expression in sympathetic neurons undergoing PCD. We find that the c-Jun protein is selectively induced in SCG neurons after NGF withdrawal, whereas the levels of the other members of the family do not change. The c-Jun protein also becomes more phosphorylated, probably on its amino terminal transactivation domain, which normally increases the ability of c-Jun to activate the transcription of target genes. By using a c-Jun dominant negative mutant, we demonstrate that AP-1 activity is essential for neuronal cell death. We also show that overexpression of c-Jun is in itself sufficient to trigger apoptosis in postmitotic neurons. These results indicate that c-Jun plays a key role in the death of sympathetic neurons after NGF withdrawal.

Results

Selective Induction of c-Jun Protein during Neuronal Cell Death

To study the pattern of expression of the Jun and Fos family of transcription factors in rat SCG neurons undergoing apoptosis, we used a set of affinity-purified rabbit polyclonal antibodies raised against the mouse Jun and Fos proteins (Pfarr et al., 1994). Each of these antibodies specifically recognizes a single member of the AP-1 family and does not cross-react with the other Jun or Fos proteins (Pfarr et al., 1994; D. Lallemand, C. Pfarr, and M. Yaniv, unpublished data). To confirm that the antibodies recognized the appropriate rat proteins, we tested them in Western blots with extracts from undifferentiated PC12 cells treated with NGF for varying lengths of time. The pattern of expression of the Jun and Fos genes has been well characterized in this rat neuronal cell line, which responds to NGF by differentiating into cells with a phenotype similar to sympathetic neurons (Bartel et al., 1989; Schlingensiepen et al., 1993; Gizang-Ginsberg and Ziff, 1994). As shown in Figure 1, each antibody recognized a PC12 protein with a similar molecular weight to the mouse protein to which it was raised. NGF treatment led to a rapid but transient increase in the level of the c-Fos protein and to the appearance of forms with a lower mobility, previously reported to be the result of increased phosphorylation (see for example, Chen et al., 1993). The level of the Fos-related protein Fra 2 also increased after NGF treatment, but this increase was long lived rather than transient. The two

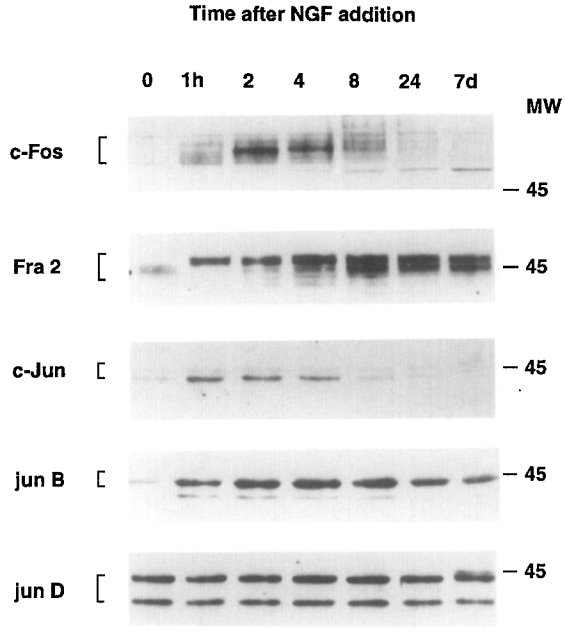

Figure 1. Pattern of Expression of the Jun and Fos Family of Proteins in NGF-Treated PC12 Cells

Undifferentiated PC12 cells were treated with NGF, and whole-cell extracts were prepared at the times shown. Extracts were fractionated by SDS-PAGE on 10% gels and transferred to nitrocellulose; 15 μg of extract was loaded per lane. The various members of the AP-1 family were detected by incubating the nitrocellulose filters with affinity-purified antibodies specific for each protein, as described in the Experimental Procedures. The identity of each rat Jun and Fos protein was verified by comparing its mobility with that of the corresponding mouse protein translated in vitro (data not shown). The brackets on the left indicate the bands corresponding to each protein. The position and size (in kilodaltons) of molecular weight markers that were run in parallel are shown on the right.

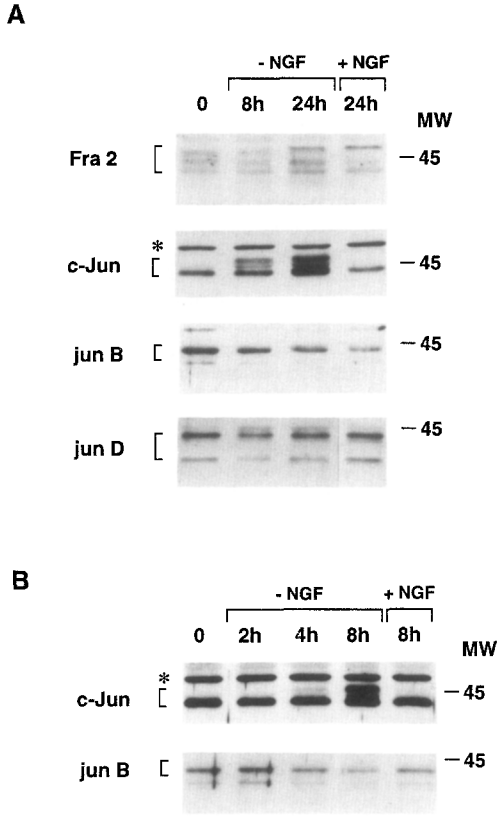

Figure 2. The c-Jun Protein Increases in Level and Becomes More Phosphorylated when SCG Neurons Are Deprived of NGF

(A) SCG neurons were cultured for 7 days in the presence of NGF. Cells were then refed with medium lacking NGF, but containing anti-NGF antibody (−NGF) or with normal NGF-containing medium (+NGF). Whole-cell extracts were prepared at the times shown and fractionated on 10% SDS-polyacrylamide gels; 7.5 μg of extract was loaded per lane. After transfer to nitrocellulose, the Fra 2, c-Jun, Jun B, and Jun D proteins were detected with affinity-purified antibodies as described in the Experimental Procedures. A nonspecific band recognized by the anti-c-Jun antibody in SCG extracts is marked with an asterisk.

(B) Whole-cell extracts were prepared 0, 2, 4, or 8 hr after NGF had been withdrawn from 7-day-old cultures of SCG neurons (−NGF) or 8 hr after the cells had been refed with NGF-containing medium (+NGF). The asterisk marks a nonspecific band detected by the anti-c-Jun antibody in SCG extracts. The brackets on the left indicate the bands that correspond to each protein. The position and size (in kilodaltons) of molecular weight markers that were run in parallel are shown on the right.

other members of the Fos family, Fos B and Fra 1, were not detected by our antibodies, which, however, did detect the mouse Fos B and Fra 1 proteins translated in vitro (data not shown). Fos B and Fra 1 may therefore be present at very low levels in PC12 cells, or it is possible that our antibodies do not recognize the rat Fos B and Fra 1 proteins. Like c-Fos, c-Jun was rapidly, but transiently, induced by NGF. Jun B was also induced by NGF but, in contrast to c-Jun, persisted at an increased level for days. Finally, as in the mouse system, the Jun D antibody recognized two bands that did not change in level after addition of NGF. In conclusion, each antibody appeared to recognize the rat homolog of the mouse protein for which it was specific, and the rat Jun and Fos proteins were induced by NGF with kinetics consistent with the published RNA data.

Having checked the specificity of our antibodies, we then determined which members of the Jun and Fos family were expressed in rat primary SCG neurons and whether their levels of expression were altered by NGF withdrawal. SCG neurons were isolated from 1-day-old rats and cultured for 7 days in the presence of NGF and antimitotic agents. On day 7, cell death was induced by switching the neurons into medium without NGF but containing neutralizing anti-NGF antibody. At various times after NGF withdrawal, both adherent and detached apoptotic neurons were harvested, and total protein extracts were prepared. To control for any effects due to the addition of fresh serum or the disturbance caused by changing the growth medium, extracts were also prepared from cells that had simply been refed with medium containing NGF (Figure 2, +NGF control). The extracts were then fractionated by SDS-PAGE, and Western blots were carried out with the various Jun and Fos antibodies (Figure 2).

Like differentiated PC12 cells (see Figure 1, 7d), SCG neurons expressed Fra 2, c-Jun, Jun B, and Jun D, but not c-Fos. Fos B and Fra 1 were also not detected. After NGF withdrawal, the levels of Fra 2, Jun B, and Jun D remained more or less constant. Furthermore, there was no increase in the levels of the Fra 2, Jun B, or Jun D proteins at timepoints earlier than 8 hr after NGF withdrawal (Figure 2B; data not shown). In contrast to the other members of the AP-1 family, the overall level of c-Jun protein increased significantly after NGF withdrawal, and there was also a dramatic change in its mobility. A more detailed analysis (Figure 2B) showed that the change in c-Jun mobility appeared to begin about 4 hr after NGF withdrawal. Again, alterations in the level and mobility of c-Jun protein were not seen in the +NGF control, indicating that these changes were the result of NGF withdrawal and were not due to the addition of fresh medium. Finally, c-Fos protein was not detected by Western blotting at 2, 4, 8, 16, or 24 hr after NGF withdrawal (data not shown), even though it could be readily detected in similar extracts from NGF-treated PC12 cells (see Figure 1). In conclusion, c-Jun was the only one of the AP-1 proteins examined that was significantly affected by NGF withdrawal.

Although inclusion of a preplating step and addition of anti-mitotic agents to the growth medium (see Experimental Procedures) kept the percentage of nonneuronal cells in our SCG cultures very low, it was still formally possible that the c-Jun protein detected in Western blots might be derived from contaminating Schwann cells or fibroblasts. To rule out this possibility, we carried out immunofluorescence experiments with the anti-c-Jun antibody (Figure 3). The c-Jun antibody weakly stained the cytoplasm and nuclei of SCG neurons maintained in the presence of NGF (Figure 3A) However, after NGF withdrawal, nuclear staining increased dramatically (Figure 3C). This increased nuclear staining first appeared at the same time that c-Jun protein levels started to increase in Western blots (data not shown). At the timepoint shown in Figure 3C (24 hr after NGF withdrawal), both normal and pyknotic nuclei

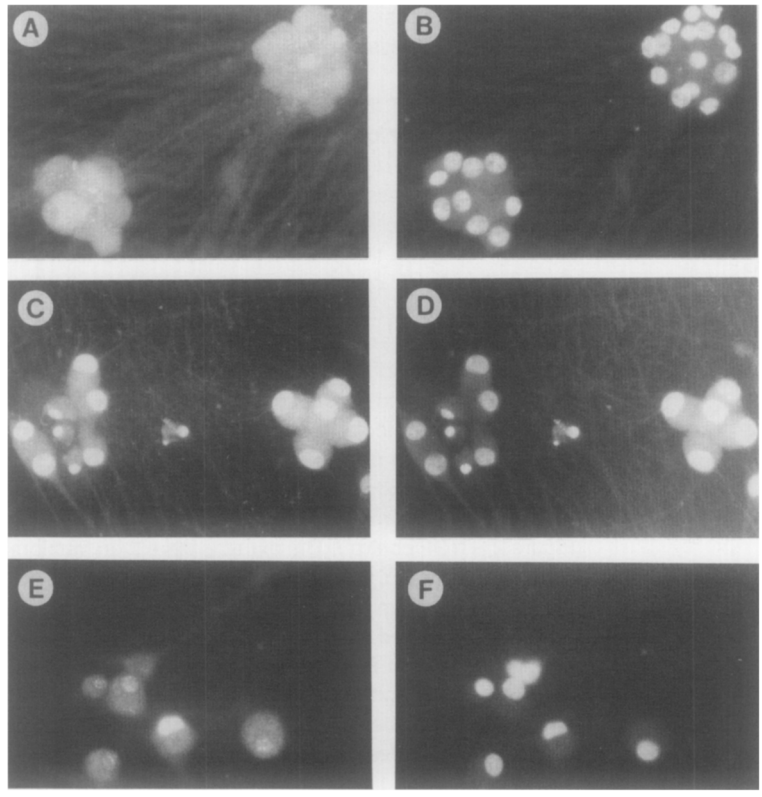

Figure 3. Immunofluorescence Analysis of c-Jun and c-Fos Expression in SCG Neurons

(A) SCG neurons were cultured for 7 days in the presence of NGF and then fixed and stained with the anti-c-Jun antibody. In the presence of NGF, weak staining of the cell body and nucleus was observed with the anti-c-Jun antibody.

(B) The cells shown in (A) stained with Hoechst.

(C) SCG neurons (7 days old) were withdrawn from NGF for 24 hr and then stained with the anti-c-Jun antibody. Both normal and pyknotic nuclei with condensed chromatin stained brightly for c-Jun.

(D) The cells shown in (C) stained with Hoechst.

(E) SCG neurons were deprived of NGF for 24 hr and then stained with the anti-c-Fos antibody. At this timepoint, less than 1% of the cells expressed c-Fos protein. Those that did expressed high levels of c-Fos and had slightly deformed nuclei. A typical example is shown.

(F) The cells shown in (E) stained with Hoechst. Immunofluorescence was carried out as described in the Experimental Procedures section. The neurons were plated on glass coverslips coated with poly-L-lysine and laminin at a density of 8000 cells per coverslip. Bar, 25 μm.

were stained, indicating that c-Jun protein levels increased before there were any visible changes in nuclear morphology, and that c-Jun protein was present in cells undergoing apoptosis.

Although we could not detect c-Fos in dying SCG neurons by Western blotting, we also investigated its pattern of expression by immunofluorescence, since it had previously been reported that expression of a c-Fos–β-galactosidase fusion protein was associated with neuronal cell death in c-*fos*–*lacZ* transgenic mice (Smeyne et al., 1993). The immunofluorescence results for c-Fos were different from those obtained for c-Jun (Figures 3E and 3F). NGF withdrawal led to the induction of c-Fos in SCG neurons, but at later timepoints only and in only a tiny percentage of the cells. For example, at 24 hr after NGF withdrawal, less than 1% of the cells expressed c-Fos protein (Figure 3E). This could explain why we did not detect c-Fos in Western blots. Furthermore, the Fos-expressing cells always had deformed nuclei, usually in the early stages of becoming pyknotic (compare Figures 3E and 3F). This suggests that, in contrast to c-Jun, c-Fos is only induced after a cell has become committed to die and at the same time as, or after, the first changes in nuclear morphology have begun.

A Dominant Negative c-Jun Mutant Blocks Neuronal Cell Death

Our Western blotting and immunofluorescence experiments demonstrated that the c-Jun protein was selectively induced when SCG neurons were deprived of NGF. To determine the role of c-Jun during neuronal cell death, we tested the effect of interfering with c-Jun function in SCG neurons by microinjecting an expression vector encoding a dominant negative c-Jun mutant, FLAGΔ169 (Figure 4A). The c-Jun deletion mutant Δ169 was derived from the mouse c-Jun cDNA by deleting sequences that encode the amino terminal c-Jun transactivation domain (amino acids 1–168). Although c-Jun Δ169 cannot activate transcription, it can still efficiently dimerize and bind to DNA (Hirai et al., 1989) and acts as a dominant negative mutant (Castellazzi et al., 1991). Δ169 will prevent any member of the Jun and Fos family from activating transcription of AP-1-dependent target genes and may do so by occupying AP-1 binding sites in the place of functional Jun/Jun or Jun/Fos dimers (see Brown et al., 1994, for a discussion of the mechanism of inhibition). We added the 8 amino acid FLAG epitope to the amino terminus of Δ169 so that we could monitor its expression in microinjected neurons by immunofluorescence with the M2 monoclonal antibody and thereby distinguish it from the endogenous c-Jun protein. The DNA sequences encoding FLAGΔ169 were cloned into pCDNA1 under the control of the powerful cytomegalovirus (CMV) immediate early promoter, which drives high level expression in a wide variety of cell types (Boshart et al., 1985).

To verify that FLAGΔ169 was efficiently expressed and correctly localized in injected neurons, we microinjected pCDFLAGΔ169 together with purified guinea pig IgG (to mark the injected cells). At 24 hr after injection, the cells were fixed and stained with antibodies against guinea pig

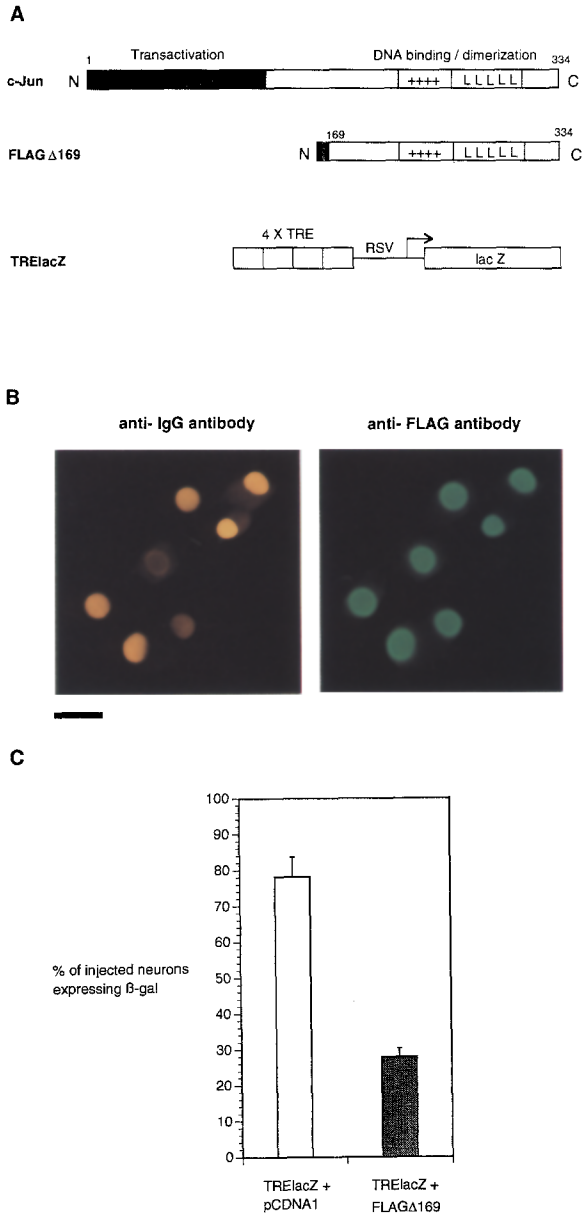

Figure 4. Structure, Pattern of Expression, and Activity of the c-Jun Dominant Negative Mutant FLAGΔ169

(A) Diagram showing the structure of FLAGΔ169 and the AP-1 reporter gene TRElacZ. The mouse c-Jun open reading frame is shown at the top. The amino-terminal transactivation domain is shaded. Plus signs represent the basic region that contacts DNA; L, the leucine zipper dimerization domain. FLAGΔ169 starts at amino acid residue 169 and therefore lacks the transactivation domain but contains the basic/leucine zipper region. The 8 amino acid FLAG epitope added to the amino terminus is represented by a closed box. The construction of FLAGΔ169 is described in the Experimental Procedures. TRElacZ contains four copies of the collagenase gene TRE cloned upstream of a Rous sarcoma virus minimal promoter linked to the E. coli β-galactosidase gene.

(B) FLAGΔ169 is efficiently expressed in microinjected SCG neurons and correctly localizes to the nucleus. SCG neurons were microinjected with pCDFLAGΔ169 at 0.05 mg/ml and guinea pig IgG at 5 mg/ml. At 24 hr after injection, the cells were fixed and stained with a rhodamine-conjugated anti-guinea pig IgG antibody (left) to detect the injected neurons and the M2 monoclonal antibody (right) to detect the FLAG epitope. The M2 monoclonal antibody was detected with an fluorescein isothiocyanate–conjugated anti-mouse IgG antibody. At left, 7 injected cells are shown. Differences in the staining intensity are the result of differences in the volume of solution injected. Guinea pig IgG present in the cytoplasm of 2 cells was the result of spillover during injection of the nucleus. All 7 injected cells expressed FLAGΔ169, which localized to the nucleus (right). Bar, 25 μm.

(C) FLAGΔ169 inhibits AP1 activity in SCG neurons. AP-1 activity was measured by injecting TRElacZ into SCG neurons. TRElacZ was injected at 0.2 mg/ml together with either pCDNA1 (as a negative control) or pCDFLAGΔ169 also at 0.2 mg/ml. The injected cells were deprived of NGF for 24 hr and then were fixed and stained with an antibody against β-galactosidase as described in the Experimental Procedures. The results are the mean ± SEM for 4 independent experiments. In each experiment, 200 neurons were injected with each injection mix.

IgG and the M2 monoclonal antibody (Figure 4B). A group of representative injected cells are shown (Figure 4B, left), all of which expressed FLAGΔ169 (Figure 4B, right). In general, FLAGΔ169 was expressed in 95% of the cells that survived injection and was always localized to the nucleus. To confirm that FLAGΔ169 inhibited AP-1 activity in SCG neurons, we injected an AP-1 reporter gene, TRElacZ, together with either the empty pCDNA1 expression vector or pCDFLAGΔ169. TRElacZ contains four AP-1 binding sites (TREs) cloned upstream of a Rous sarcoma virus minimal promoter linked to the Escherichia coli β-galactosidase gene (Figure 4A; Alberts et al., 1993). After injection, the neurons were switched into medium without NGF for 24 hr and then were fixed and stained with an anti-β-galactosidase antibody. The number of cells expressing β-galactosidase was determined, and the result is shown in Figure 4C. Expression of FLAGΔ169 reduced the percentage of β-galactosidase-positive cells by 3-fold relative to the population injected with pCDNA1, indicating that it does indeed act as a dominant inhibitor of AP-1 activity in dying SCG neurons.

We next investigated whether the expression of FLAG Δ169 in SCG neurons could prevent or slow cell death after NGF withdrawal. In these experiments we compared the c-Jun dominant negative mutant with pCDNA1, which should not alter the time course of death, and with pCDbcl-2, a Bcl-2 expression vector. The latter served as a positive control, since overexpression of Bcl-2 has previously been shown to slow the rate of death of sympathetic neurons deprived of NGF (Garcia et al., 1992). The expression vectors were injected into 4- to 5-day-old SCG neurons together with Texas Red dextran to mark the injected cells. After injection, the cells were left overnight to allow time for the injected DNAs to be expressed and then were refed with medium lacking NGF and containing anti-NGF antibody. Immediately after the medium change, the number of Texas Red–positive cells was determined. The cells were deprived of NGF for 2 or 3 days, and calcein AM was then added to the medium to mark viable cells. The percentage of viable cells at 2 or 3 days after NGF withdrawal was then calculated as described in the Experimental Procedures section. The outcome of these experiments is shown in Figure 5. After NGF withdrawal, neurons injected with pCDbcl-2 showed increased survival compared with those injected with the empty expression vector pCDNA1 or with Texas Red dextran alone, a result consis-

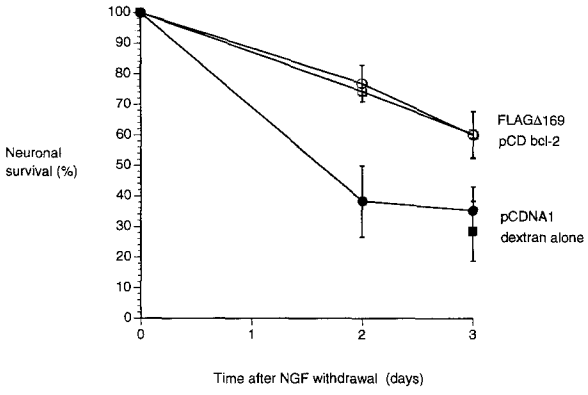

Figure 5. The c-Jun Dominant Negative Mutant FLAGΔ169 and Bcl-2 Each Protect Sympathetic Neurons against Cell Death after NGF Withdrawal

SCG neurons (4–5 days old) were injected with pCDFLAGΔ169 or pCDbcl-2 or pCDNA1 at 0.05 mg/ml together with Texas Red dextran at 20 mg/ml (to mark the injected cells). Several hours later, the injected cells were refed with medium lacking NGF, but containing anti-NGF antibody. Immediately after refeeding, the number of neurons that had survived injection was determined. At 2–3 days later, the percentage of viable, injected neurons that remained was determined by staining with calcein AM, as described in the Experimental Procedures section. Each point represents the mean ± SEM for three independent experiments. In each experiment, 200 neurons were injected with each construct. Open circles, pCDFLAGΔ169; open squares, pCDbcl-2; closed circles, pCDNA1; closed squares, Texas Red dextran alone.

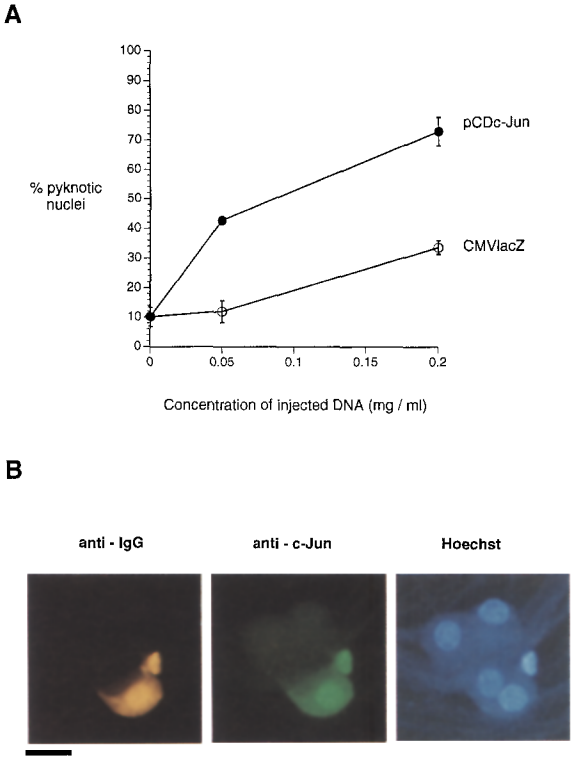

Figure 6. Overexpression of c-Jun Alone Is Sufficient to Induce Apoptosis in SCG Neurons

(A) SCG neurons that had been cultured in the presence of NGF for 7 days were injected with pCDc-Jun or CMVlacZ at 0.05 or 0.2 mg/ml together with guinea pig IgG at 5 mg/ml (to mark the injected cells). After injection, the cells were refed with NGF-containing medium and left for 4 days. The neurons were then fixed and stained with an antibody against guinea pig IgG and antibodies against c-Jun or β-galactosidase. Nuclear morphology was visualized by Hoechst staining. The percentage of pyknotic nuclei was then determined for cells overexpressing c-Jun or β-galactosidase and for uninjected cells. Only nuclei that were unequivocally pyknotic (i.e., those with highly condensed chromatin or that were grossly distorted or fragmented) were scored as pyknotic. For injections at 0.05 mg/ml, the results shown are the mean of 3 independent experiments ± SEM. For injections at 0.2 mg/ml, the results are the mean of 5 independent experiments. Closed circles, pCDc-Jun; open circles, CMVlacZ.

(B) Morphology of injected neurons overexpressing c-Jun. Two representative cells are shown. The injected cells were marked by including guinea pig IgG in the injection mix (left). Hoechst staining (right) shows that one has a normal nucleus, as can be seen by comparing it with the uninjected cells nearby. The other c-Jun-expressing cell has a shrunken, crescent-shaped nucleus with condensed chromatin. Bar, 17 μm.

tent with the findings of Garcia et al. (1992). Importantly, injection of pCDFLAGΔ169 also led to a higher percentage of surviving cells. In fact, FLAGΔ169 was as effective as Bcl-2 in protecting the injected neurons from PCD. Furthermore, the viable neurons expressing FLAGΔ169 had a similar morphology to those saved by Bcl-2 (data not shown). This result indicates that AP-1 activity is rate limiting for neuronal cell death after NGF withdrawal, since death can be inhibited by overexpressing a c-Jun dominant negative protein. Since c-Jun is the only member of the Jun and Fos family that significantly increases in level after SCG neurons are deprived of NGF, it is likely that c-Jun is an important component of the form of AP-1 essential for the resulting cell death.

Having demonstrated that c-Jun protein levels increase after NGF withdrawal and that AP-1 activity is essential for this form of cell death, we wondered whether overexpression of c-Jun alone is sufficient to cause the death of SCG neurons. We therefore injected neurons with an expression vector for the full-length c-Jun protein (pCDc–Jun) or with a β-galactosidase expression vector (CMVlacZ) as a negative control. After injection, the neurons were maintained in the presence of NGF for 4 days and then were fixed and stained with the anti-c-Jun antibody or with a monoclonal antibody against β-galactosidase to detect cells overexpressing c-Jun or β-galactosidase, respectively. Nuclear morphology was visualized by Hoechst staining, and the percentage of pyknotic nuclei was determined for cells expressing c-Jun or β-galactosidase. At 4 days after injection, 10% of the uninjected cells had pyknotic nuclei (Figure 6A). When CMVlacZ was injected at a concentration of 0.05 mg/ml, 12% of the cells expressing β-galactosidase had pyknotic nuclei. In contrast, 43% of the cells injected with pCDc–Jun at an equivalent concentration were pyknotic, a 3.5-fold increase over the background level in cells injected with the CMVlacZ control vector. When CMVlacZ was injected at a higher concentration (0.2 mg/ml), 33% of the cells had pyknotic nuclei, a 3-fold increase compared with the uninjected cells. This was presumably a nonspecific effect due to expression of high levels of β-galactosidase or the presence of the strong CMV promoter. Importantly, however, 73% of the cells

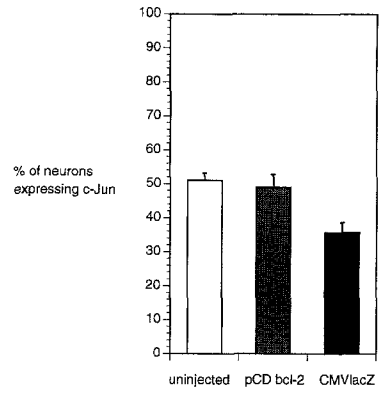

Figure 7. Overexpression of Bcl-2 Does Not Prevent Induction of c-Jun after NGF Withdrawal

SCG neurons were injected with pCDbcl-2 or CMVlacZ at 0.05 mg/ml and were then refed with medium lacking NGF but containing anti-NGF antibody. At 24 hr later, the cells were fixed and stained with antibodies against Bcl-2 or β-galactosidase and the anti-c-Jun antibody. The percentage of neurons expressing Bcl-2 or β-galactosidase that also expressed c-Jun was then determined. Only those cells in which c-Jun staining was clearly above background were scored as c-Jun positive. The results shown are the mean of 3 independent experiments ± SEM. In each experiment, 200 neurons were injected with each construct.

injected with pCDc–Jun at 0.2 mg/ml were pyknotic, a 2.2-fold increase over the background level in cells injected with CMVlacZ (Figure 6A). The morphology of two representative cells overexpressing c-Jun is shown in Figure 6B. The injected cells were marked by including guinea pig IgG in the injection mix (Figure 6B, left); c-Jun staining is shown in the center panel. Hoechst staining (Figure 6B, right) shows that one cell has a normal nucleus, as can be seen by comparing it with the uninjected cells nearby. The other c-Jun-expressing cell has a shrunken, crescent-shaped nucleus with condensed chromatin. In general, the level of c-Jun expressed in cells injected with pCDc-Jun was similar to, or higher than, that observed in SCG neurons after NGF withdrawal (data not shown). In conclusion, these results indicate that as well as c-Jun being essential for neuronal cell death, overexpression of c-Jun is in itself sufficient to trigger apoptosis in SCG neurons and can override the protective effect of NGF.

Finally, we wondered whether Bcl-2 might protect SCG neurons against PCD by blocking induction of c-Jun. We therefore injected neurons with pCDbcl-2 or, as a negative control, with CMVlacZ. After injection, the neurons were withdrawn from NGF for 24 hr and then were fixed and stained with monoclonal antibodies against Bcl-2 or β-galactosidase and the anti-c-Jun polyclonal antibody. The results of this experiment are shown in Figure 7. At 24 hr after removal of NGF, 50% of the uninjected cells expressed c-Jun. When cells were injected with CMVlacZ, this figure was slightly reduced. In the case of neurons injected with pCDbcl-2, there was no reduction in the percentage of cells expressing c-Jun protein, demonstrating that Bcl-2 does not promote the survival of NGF-deprived SCG neurons by inhibiting induction of c-Jun.

Discussion

We have used antibodies specific for each member of the Jun and Fos family of transcription factors to study their pattern of expression in sympathetic neurons undergoing PCD. We found that sympathetic neurons express Fra 2, c-Jun, Jun B, and Jun D. However, after NGF withdrawal, c-Jun was the only one of these proteins whose pattern of expression changed significantly. Two effects were evident; first, c-Jun protein levels increased substantially, whereas the levels of Fra 2, Jun B, and Jun D remained relatively constant; second, the mobility of the c-Jun protein changed, starting about 4 hr after NGF withdrawal. Although we could not detect c-Fos in dying SCG neurons by Western blotting, we found in immunofluorescence experiments that at late time points after NGF withdrawal, a few cells (less than 1% at 24 hr) expressed high levels of c-Fos protein. These cells always had pyknotic nuclei, suggesting that induction of c-Fos occurred at the same time as, or after, the first changes in nuclear morphology had taken place. Our results are therefore consistent with those of Smeyne et al. (1993), who observed that c-Fos expression is associated with certain forms of neuronal cell death in vivo. However, in the neurons we have studied, c-Fos induction appears to be a relatively late event, which either occurs transiently in all dying neurons, or which only occurs in a subset of dying neurons. In contrast, we detected c-Jun protein in both normal and pyknotic nuclei, and it appeared to be induced much earlier than c-Fos. Since 50% of SCG neurons are committed to die by 22 hr after NGF withdrawal (Deckwerth and Johnson, 1993), it seems that c-Jun is induced before, and c-Fos after, a neuron has become committed to die.

The increase in c-Jun protein levels that we have observed might be the result of increased transcription of the c-*jun* gene. The c-Jun protein positively autoregulates its own expression and is permanently bound to two variant TRE sequences in the c-*jun* promoter as c-Jun/activating transcription factor 2 heterodimers, whose activity is regulated by phosphorylation (Angel et al., 1988; Herr et al., 1994). Stimuli, such as ultraviolet treatment, that increase the rate of transcription of the c-*jun* gene do so by activating protein kinases that phosphorylate the c-Jun transcriptional activation domain on serines 63 and 73. This has been shown to increase the ability of c-Jun to activate the transcription of target genes, including the c-*jun* gene itself (Pulverer et al., 1991; Smeal et al., 1991, 1992; Karin, 1994). Interestingly, we observed that when SCG neurons undergo apoptosis, the apparent mobility of the c-Jun protein in SDS-PAGE decreases. Previously, this change was found to correlate with increased phosphorylation of the c-Jun transcriptional activation domain on serines 63 and 73 (Pulverer et al., 1991; Kyriakis et al., 1994). A family of Jun amino terminal kinases, also known as stress-activated protein kinases, which directly bind and phosphorylate the c-Jun transactivation domain, has recently been identified, and several members have now been cloned (Hibi et al., 1993; Dérijard et al., 1994; Kyriakis et al., 1994). Although related to MAP kinases,

the Jun kinases preferentially phosphorylate c-Jun and are activated by different stimuli, notably ultraviolet radiation, tumor necrosis factor α, ceramide, cellular stress, and reactive oxygen species (Davis, 1994), all treatments that can cause cell death. It is tempting to speculate that NGF withdrawal leads to some form of cellular stress, perhaps an increase in reactive oxygen species, which causes the activation of Jun kinase. Thus, the change in the mobility of c-Jun that we have observed would be consistent with phosphorylation by a member of the Jun kinase family, starting about 4 hr after NGF withdrawal. Activation of c-Jun by phosphorylation would lead to activation of the c-*jun* promoter, which might account for the increase in c-Jun protein levels that we have observed.

What might be the role of the c-Jun protein induced in dying SCG neurons? There are several possibilities. For example, c-Jun might cause cell death by activating the transcription of target genes whose protein products are either effectors or triggers of the cell death program. Alternatively, c-Jun activation might be the result of certain physiological changes that occur after NGF withdrawal, and c-Jun might activate genes that encode proteins that counteract these physiological changes; i.e., c-Jun might orchestrate a protective or homeostatic response. Finally, a number of studies have reported a delayed and prolonged induction of c-Jun in peripheral sensory neurons or motor neurons after axon damage. Since c-Jun remained at elevated levels until the nerve had fully regenerated, it was suggested that in this context c-Jun might be involved in regeneration (see, for example, Gold et al., 1993 and De Felipe and Hunt, 1994). To distinguish among these different possibilities, we tested the effect of overexpressing a transactivation-defective c-Jun dominant negative mutant in SCG neurons. We found that this mutant blocked AP-1 activity in SCG neurons (see Figure 4) and protected them against PCD as effectively as Bcl-2 did (see Figure 5). Even though FLAGΔ169 does not distinguish among different members of the Jun and Fos family, we can safely conclude that AP-1 activity is essential for the death of SCG neurons after NGF withdrawal. However, we believe that c-Jun plays a key role because, first, it is the only member of the family whose level significantly increases after NGF withdrawal; second, it is induced in all of the SCG neurons before they are irreversibly committed to cell death; and, third, its overexpression is sufficient to induce apoptosis in SCG neurons. How might c-Jun function in this situation? One possibilty is that it forms heterodimers with Fra 2, which is expressed in sympathetic neurons, or with other proteins, such as activating transcription factor 2. Alternatively, as has previously been demonstrated, c-Jun might act on its own as a homodimer. Our results do not exclude the possiblility that c-Jun might also be involved in regeneration in vivo, especially since our experiments were carried out with purified cultures of neurons isolated from their normal environment. Indeed, c-Jun might simultaneously activate genes that cause cell death and genes involved in regeneration, the outcome perhaps being determined by interactions with other neurons or glial cells.

While this manuscript was in preparation, Estus et al. (1994) reported similar results in sympathetic neurons. Using the technique of reverse transcription-PCR (RT-PCR), these authors found that the c-Jun, c-Fos, and Fos B mRNAs increased in level after NGF withdrawal, and that microinjection of an anti-c-Jun antibody inhibited cell death. Our findings, which were obtained by a different approach, confirm and extend their results. In contrast to Estus et al., we used antibodies specific for each member of the Jun and Fos family to study their pattern of expression in SCG neurons. In Western blotting and immunofluorescence experiments, we have demonstrated that the changes in RNA level detected by Estus et al. actually lead to changes in protein level. Furthermore, we have also been able to detect a posttranslational modification of the c-Jun protein, which suggests that a signal transduction pathway that activates Jun kinase may play a role in neuronal cell death. Estus et al. found that the level of c-Jun RNA increases about 5 hr after NGF withdrawal, which is also the time at which we first observed increased phosphorylation of c-Jun. These results are entirely consistent with a model in which increased phosphorylation of c-Jun by a member of the Jun kinase family leads to transcriptional activation of the c-*jun* promoter. Estus et al. also demonstrated by RT-PCR that the c-Fos mRNA increases in level after NGF withdrawal. In contrast, we were unable to detect c-Fos protein in dying SCG neurons by Western blotting, although our antibody readily detected c-Fos when it was present, for example, in NGF-treated PC12 cells (see Figure 1). We did, however, find a small number of Fos-expressing cells when we used the same antibody in immunofluorescence. These results suggest that although the c-Fos RNA could be detected by the extremely sensitive technique of RT-PCR, relatively few dying SCG neurons actually expressed detectable c-Fos protein. By injecting antibodies against various members of the AP-1 family, Estus et al. demonstrated that c-Jun and members of the Fos family are essential for cell death. Our observation that a transactivation-defective c-Jun dominant negative mutant blocks the death of SCG neurons is consistent with their results and, in addition, demonstrates that c-Jun and its partners from the Fos family cause cell death by binding to DNA and activating transcription. We strengthen this argument by showing that overexpression of c-Jun alone can trigger cell death in neurons. Finally, Estus et al. speculated that induction of c-Jun might be the result of oxidative stress after NGF withdrawal. Since it has been suggested that Bcl-2 may block oxidative damage (Hockenbery et al., 1993), they proposed that Bcl-2 might prevent neuronal cell death by inhibiting induction of c-Jun. However, we have found that this is not the case. By injecting neurons with a Bcl-2 expression vector and carrying out immunofluorescence with an anti-c-Jun antibody, we found that NGF withdrawal led to c-Jun induction, even when Bcl-2 was overexpressed (see Figure 7). We propose that Bcl-2 acts downstream of c-Jun, perhaps by inactivating cytosolic cell death effector proteins. This would be consistent with the observations of Jacobson et al. (1994), who found that

Bcl-2 expression can protect cytoplasts against cell death induced by survival factor withdrawal or by treatment with the broad spectrum kinase inhibitor staurosporine.

Our results provide a further example of the usefulness of c-Jun dominant negative mutants for investigating the biological functions of members of the Jun and Fos family. Mutants similar to FLAGΔ169 have been used to show that AP-1 activity is important for cell proliferation (Castellazzi et al., 1991), necessary for Ras to transform fibroblasts (Lloyd et al., 1991), and that Drosophila Jun mediates Ras-dependent photoreceptor determination (Bohmann et al., 1994). Identification of genes that are essential for mammalian cell death has been hampered by the lack of a suitable genetic system. However, the microinjection of neurons with vectors that express naturally occurring inhibitors such as crmA, a viral inhibitor of interleukin 1β–converting enzyme (ICE) family proteases, has provided an alternative approach (Gagliardini et al., 1994). The use of dominant negative mutants such as the c-Jun dominant negative described in this paper represents another strategy that could be used in the future for studying the role of other transcription factors in neuronal cell death or to dissect the signal transduction pathway that leads to the induction of c-Jun in dying sympathetic neurons. It would also be interesting to study the role of c-Jun in neuronal cell death in vivo. Although the mouse c-*jun* gene has been disrupted by homologous recombination, the resulting c-*jun* (-/-) mice had a complex phenotype and died during embryonic development at about 12.5 days (Johnson et al., 1993; Hilberg et al., 1993), thus precluding an analysis of the role of *c-jun* in the developmental death of sympathetic neurons, which occurs after birth. However, the creation of transgenic mice in which FLAGΔ169 is expressed under the control of a promoter specific for postmitotic neurons, such as that of the neuron-specific enolase gene (Forss-Petter et al., 1990), would provide an alternative approach. It would also enable us to address the question of whether AP-1 activity is necessary for neuronal regeneration in vivo.

Our results demonstrate that c-Jun is essential for the PCD of sympathetic neurons. What role might c-Jun play during this process? A simple hypothesis would be that, following NGF withdrawal, c-Jun might activate the transcription of genes encoding proteins essential for cell death, such as members of the CED-3/ICE family of proteases. Before NGF withdrawal, such proteins might be present at limiting concentrations, and an increase in their levels might override the protective effect of cell death suppressors such as the Bcl-2 protein. Consistent with such a model, artificially increasing the concentration of ICE in DRG neurons by overexpression was found to cause apoptosis (Gagliardini et al., 1994). Furthermore, apoptosis of mammary epithelial cells induced by loss of contact with the extracellular matrix was found to be associated with an increase in the level of ICE RNA and required the activity of ICE family proteases (Boudreau et al., 1995). Arguments against a model in which genes encoding cell death effectors are activated during cell death have been presented by Jacobson et al. (1994) and were based on the observation that cells can undergo apoptosis in the absence of a nucleus. These authors concluded that the cell death effector proteins must be cytosolic, preexist in all cells, and can be activated posttranslationally. Though this is certainly true in those situations in which cell death does not require new transcription or protein synthesis, it does not exclude the possibility that in other situations the cell death machinery may be activated by increasing the level of cell death effectors through new synthesis.

An alternative hypothesis, which we favor, is that c-Jun might be essential for cell death because it activates genes involved in cell proliferation. We have previously hypothesized that the cell death that follows the withdrawal of NGF from postmitotic neurons may be the result of an abortive attempt to reenter the cell cycle (Rubin et al., 1993, 1994). According to this model, removal of NGF would lead to the activation of cyclin-dependent kinases or other proteins that drive cell cycle progression. In the context of a terminally differentiated neuron that has withdrawn from the cell cycle, such enzymes might function directly as cell death effector proteins; i.e., components of the cell cycle machinery would be employed in the cell death program. Alternatively, the inappropriate activation of such enzymes in postmitotic neurons might trigger the cell death program. Evidence to support this type of idea has come from a recent study by Freeman et al. (1994). These authors used RT-PCR analysis to investigate whether cell cycle genes are expressed in sympathetic neurons and to determine whether any of these are induced by NGF withdrawal. Though the level of most of the transcripts they studied decreased in dying neurons, the cyclin D1 mRNA was selectively induced, peaking at 15–20 hr after NGF withdrawal, the time when neurons become committed to die. However, it was not demonstrated whether cyclin D1 was essential for cell death or whether a cyclin D1–dependent kinase was activated. Interestingly, the cyclin D1 gene is a potential target of c-Jun transactivation, since the cyclin D1 promoter contains potential AP-1 binding sites and can be activated by overexpression of c-Jun in transient transfection experiments (Herber et al., 1994). Consistent with such an idea, c-Jun protein levels start to increase 4–8 hr after NGF withdrawal (see Figure 2) before cyclin D1 levels peak (15–20 hr). Freeman et al. also showed that SCG neurons do not express CDK2, cyclin A, or CDC2, all of which are required for normal cell cycle progression. Therefore the transcriptional activation of proto-oncogenes, such as c-Jun and cyclin D1, which would act as a strong growth-promoting signal, in cells that are locked out of the cell cycle might be the event that triggers apoptosis.

In conclusion, we have demonstrated that the c-Jun protein is selectively induced in sympathetic neurons undergoing PCD, and by microinjecting a dominant negative c-Jun expression vector we have shown that AP-1 activity is essential for this form of cell death. Future experiments will be directed toward identifying Jun target genes and toward determining the mechanisms that are responsible for the increase in the level of c-Jun protein and for its

increased phosphorylation. Our results also show that, in addition to playing an important role in cell proliferation and differentiation, c-Jun may also be involved in cell death. Future work will determine whether this is true for other kinds of neurons and cell types.

Experimental Procedures

Cell Culture

Sympathetic neurons from the superior cervical ganglia of 1-day-old Sprague-Dawley rats were isolated and cultured as described by Johnson and Argiro (1983) with certain modifications. In brief, after dissection, the ganglia were desheathed in L15 medium plus 0.1% fetal calf serum plus penicillin/streptomycin and then dissociated by treatment with a 1:1 mixture of 0.4% collagenase/trypsin–EDTA and by trituration, using a syringe and 19G and 23G needles. Nonneuronal cells were then removed by plating the cell suspension in 10 ml of growth medium (for up to 30 ganglia) in an uncoated 9 cm dish for 2.5 hr. The resulting supernatant enriched in SCG neurons was then plated on 3.5 cm diameter dishes coated with poly-L-lysine and collagen in DMEM containing 10% fetal calf serum, 2 mM glutamine, penicillin/streptomycin, and NGF at 50 ng/ml. Collagen was prepared from fresh rat tails. NGF (2.5 S) was provided by S. Brooks and purified from adult male mouse submaxillary glands as described (Suda et al., 1978). The antimitotic agents fluorodeoxyuridine and uridine were added to a final concentration of 20 μM to limit the proliferation of nonneuronal cells. The neurons were usually maintained for 6–8 days in the presence of NGF before being used for cell death experiments. NGF withdrawal was carried out by changing the medium to DMEM/10% fetal calf serum lacking NGF and containing anti-NGF antibody at 100 ng/ml (Boehringer Mannheim). For immunofluorescence and microinjection experiments, neurons were plated on glass coverslips coated with poly-L-lysine and laminin. PC12 cells were cultured in SATO medium as described by Doherty et al. (1988) at a density of 2×10^6 cells per dish in 9 cm dishes coated with poly-L-lysine and collagen.

Western Blot and Immunofluorescence Analysis

Cell extracts for Western blotting were prepared as follows. The growth medium from the neuronal cultures was removed but kept, so that any detached, apoptotic cells would be included in the analysis. The attached neurons that remained were then harvested by scraping in a small volume of ice cold PBS. Adherent and floating cells were pooled and pelleted by centrifugation at 2000 rpm for 5 min at 4°C in a bench centrifuge. The cell pellet was resuspended in 1 ml of ice cold PBS and transferred to a microfuge tube. The cells were then repelleted and resuspended in 50–200 μl of SDS lysis buffer (10 mM Tris-HCl [pH 7.6], 150 mM NaCl, 0.5 mM EDTA, 1 mM EGTA, 1% SDS) containing a cocktail of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, 5 μg/ml leupetin, 2 μg/ml aprotinin) and heated at 90°C for 20 min. The lysate was then centrifuged for 20 min in a microfuge at 4°C. The final supernatant was transferred to a fresh tube, snap frozen in dry ice, and stored at −80°C. For Western blots, proteins were separated on 10% SDS-PAGE gels using the Bio-Rad mini Protean II electrophoresis system. Extract was usually loaded at 7.5–15 μg per lane. After SDS-PAGE, the proteins were blotted onto Hybond-ECL nitrocellulose using a Bio-Rad minitransblot electrophoretic transfer cell. Different members of the Jun and Fos family were detected with affinity purified rabbit polyclonal antibodies as described by Pfarr et al. (1994) using a horseradish peroxidase–conjugated anti-rabbit secondary antibody and the ECL system (Amersham). The Jun and Fos antibodies were raised against fusions between glutathione-S-transferase and small portions of the coding sequences from the mouse Jun and Fos proteins and were affinity purified (Pfarr et al., 1994; D. Lallemand et al., unpublished data).

For immunofluorescence experiments, neurons grown on glass coverslips coated with poly-L-lysine and laminin were rinsed in PBS, fixed in 3% paraformaldehyde for 20 min at room temperature, rinsed again with PBS, and permeabilized with 0.5% Triton X-100 in PBS for 5 min at room temperature. Blocking and antibody incubations were then carried out by standard procedures (Harlow and Lane, 1988). Primary and secondary antibodies were diluted in 1% bovine serum albumin in PBS. To detect c-Jun in immunofluorescence experiments, an affinity-purified rabbit polyclonal antibody was used (Pfarr et al., 1994). β-galactosidase was detected with a monoclonal antibody from Promega, diluted 1:400. FLAGΔ169 was detected with the M2 monoclonal antibody (IBI Kodak) diluted 1:200. Bcl-2 was detected with a monoclonal antibody (clone 124) diluted 1:80 (DAKO). Fluorescein-conjugated goat anti-mouse and anti-rabbit secondary antibodies were from Jackson Labs and were diluted 1:100. After the secondary antibody incubation, the cells were rinsed in PBS and the nuclei stained with Hoechst dye (H 33342) at 1 μg/ml in water and then given a final rinse with water. Coverslips were mounted in Citifluor. The slides were viewed on a Nikon Microphot FXA fluorescence microscope. Kodak ektachrome panther 400X and TMAX (TMY400) films were used for color and black and white photographs, respectively.

Plasmid Constructions

Expression Vectors

The murine c-Jun and *bcl-2* cDNAs and the c-Jun dominant negative mutant FLAGΔ169 were all cloned into the expression vector pCDNA1 (Invitrogen) for microinjection experiments. In pCDNA1, transcription is driven by the strong CMV immediate early promoter. To construct pCDc-Jun, a HindIII–PstI fragment containing the leader sequence of the β-globin RNA upstream of the murine c-Jun coding sequence was subcloned into pCDNA1 from the plasmid T7 β c-Jun (Hirai et al., 1989). pCDFLAGΔ 169 was constructed in two steps. The plasmid T7 β c-Jun Δ169 (Hirai et al., 1989) was digested with NcoI and PstI, treated with phosphatase, and the fragment containing the vector and β-globin leader sequence was purified by agarose gel electrophoresis. This vector fragment was then ligated to a double-stranded linker oligonucleotide with NcoI and AccI ends containing an optimized ATG followed by the 8 amino acid FLAG epitope (AspTyrLysAspAspAspAspLys) and c-Jun sequences from amino acid 169 to 172, together with an AccI–PstI fragment containing c-Jun sequences from amino acid 173 to 334. This generated the plasmid T7 β FLAGΔ169. The sequence of the linker oligo was as follows: 5′-CATGGACTACAAGGACGACGACGACAAGGAGCCTCCGGT-3′ (upper strand) and 5-AGACCGGAGGCTCCTTGTCGTCGTCCTTGTAGTC-3′ (lower strand). The HindIII–PstI fragment from T7 β FLAGΔ169 containing the FLAG-tagged c-Jun Δ169 deletion mutant was then subcloned into pCDNA1. pCDbcl-2 was constructed by subcloning a HindIII–EcoRI fragment containing the murine *bcl-2* cDNA (Nunez et al., 1990) into pCDNA1. The structure of all plasmid constructs was verified by direct sequencing using a primer that hybridized to the T7 promoter in pCDNA1 and a USB Sequenase version 2.0 kit. The expression vectors were also transcribed in vitro with T7 polymerase and the resulting RNAs translated in a TnT rabbit reticulocyte lysate system (Promega) to check that proteins of the correct size were made. CMV lacZ contains the E. coli β-galactosidase gene under the control of the CMV immediate early promoter (Alberts et al., 1994).

Reporter Genes

The AP-1 reporter gene TRElacZ contains four copies of the collagenase gene TRE cloned upstream of a Rous sarcoma virus minimal promoter linked to the E. coli β-galactosidase gene (Alberts et al., 1993).

Microinjection

For microinjection, neurons were plated on glass coverslips coated with poly-L-lysine and laminin. Microinjection was carried out using a Zeiss Axiovert 135M microscope with an Eppendorf transjector (5171) and micromanipulator (5246). Microinjection needles were pulled from glass capillaries using a horizontal electrode puller (Camden Instruments, Model 773) and loaded using Eppendorf microloaders. DNA was injected directly into the nucleus. The expression vectors CMVlacZ, pCDc-Jun, pCDFLAGΔ169, and pCDbcl-2 were injected in 0.5× PBS at a concentration of 0.05–0.2 mg/ml. The reporter gene TRElacZ was injected at 0.2 mg/ml. Approximately 40%–80% of SCG neurons survived microinjection. Immunofluorescence analysis was carried out to verify that β-galactosidase, c-Jun, FLAGΔ169, or Bcl-2 protein were expressed and correctly localized within neurons after injection of the appropriate expression vectors. For these experiments, purified guinea pig IgG (Sigma) was added to the injection mix at a final concentration of 5 mg/ml. After injection, the neurons were fixed and stained with a rhodamine-conjugated donkey anti-guinea pig IgG antibody (Jackson Labs) diluted 1:100 to identify injected cells and

anti-β-galactosidase, anti-c-Jun, anti-Bcl-2, or the M2 monoclonal antibody, which detects FLAG-tagged proteins, as described above. In all cases, approximately 90% of the neurons that had survived injection expressed the appropriate protein.

For analyzing the effect of expression vectors on neuronal survival in the absence of NGF, neurons were plated at a density of 8000 per coverslip and used 4–5 days after plating. Neutral 70,000 MW Texas Red dextran (Molecular Probes) was used to mark the injected cells in these experiments and was added to the injection mix at a final concentration of 20 mg/ml. After injection, the neurons were left overnight and then refed with medium lacking NGF and containing the anti-NGF antibody. The number of Texas Red dextran–positive cells that had survived injection was then determined. The medium was changed daily, and 2–3 days after NGF withdrawal, calcein AM (Molecular Probes) was added to the medium. This is converted by the esterase activity in viable cells to calcein, which fluoresces green. Cell viability was assessed by counting the total number of Texas Red–positive cells and by noting their morphology and whether they were green (viable) or not. The microinjection experiments were independently carried out by more than one investigator, and on a number of occasions the slides were counted in a blinded fashion.

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